

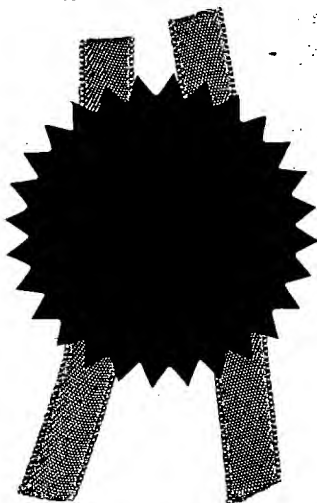
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Application for Patent

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אני, (שם המבקש, מענו ולגבי גוף מאגד - מקום התאגדותו)  
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חברה רשומה בישראל  
ת.ד. 95, רחובות

בעל אמצאה מכח העברה Assignment  
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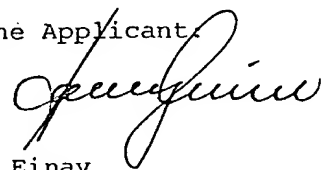
(בעברית)  
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Antibodies to anti-cytotoxic proteins

(באנגלית)  
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hereby apply for a patent to be granted to me in respect thereof.

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* בקשת חלוקה - Application of Division		* בקשת פטנט מוסף - Application for Patent Addition		* דרישה רין קדימה Priority Claim	
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94039

Antibodies to anticytotoxic proteins

נוגדנים לחלבונים אנטי-ציטוטוקסיים

Yeda Research & Development Co. Ltd.

T/820

This invention relates to antibodies against the two Tumor Necrosis Factor (TNF) Binding Proteins designated TBP-I and TBP-II which were described in prior patent applications No. 83878 and No. 90339, respectively, of the same applicants. It further relates to the ability of said antibodies to block the binding of TNF to cells, the two antibodies affecting differently cells of different kinds. It relates also to the use of those antibodies and of F(ab) fragments thereof, and salts, functional derivatives or active fractions of said antibodies and fragments, as pharmaceutical agents both for mimicking and blocking effects of TNF on part or all cells in the human body.

Another aspect of the invention features the diagnostic use or tests for measuring either the TBPs or the antisera against them, based on determining the interaction of the antibodies with the TBPs. These diagnostic uses are of two kinds: (a) detecting endogenously produced antibodies to TBPs in body fluids, to determine the extent to which such antibodies, by mimicking or blocking the effects of TNF, contribute to pathological manifestations of diseases, and (b) quantifying the levels of TBPs in body fluids to detect or measure over- or under-production of these proteins in any disorder characterized by abnormal production of said proteins.

The proteins TBP-I and TBP-II, which specifically bind tumor necrosis factor, were isolated from human urine by affinity purification on a column of immobilized TNF, followed by reverse phase high performance liquid chromatography (HPLC). The apparent molecular weights of the two proteins in sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) were similar (about 30000). Both proteins provided protection against the in vitro cytotoxic effect of TNF and both bound lymphotoxin or TNF- $\beta$  less effectively than tumor necrosis factor or TNF- $\alpha$ . However, the two proteins were found immunologically distinct and had differing N-terminal amino acid sequences: Asp-Ser-Val-Cys-Pro... for TBP-I and Val-Ala-Phe-Thr-Pro... for TBP-II. The N-terminal sequence of TBP-I was homogeneous while that of TBP-II was truncated, to varying extent.

Tumor Necrosis Factor or TNF- $\alpha$  and lymphotoxin or TNF- $\beta$  (hereinafter, TNF refers to both TNF- $\alpha$  and TNF- $\beta$ ) are cytokines which have many effects on cells. Both TNF- $\alpha$  and TNF- $\beta$  initiate their effects by binding to specific cell surface receptors. Some of the effects, such as destruction of tumor cells or virus-infected cells, are likely to be beneficial to the organism and to contribute to defense against infectious agents and recovery from injury. However, both TNF- $\alpha$  and TNF- $\beta$  also have effects which can be extensively deleterious. Over-production of TNF- $\alpha$  can play a major pathogenic role in several diseases. Thus, effects of TNF- $\alpha$ , primarily on the vasculature, are known to be a major cause for symptoms of septic shock. In some diseases, TNF may cause excessive loss of weight (cachexia) by suppressing activities of adipocytes and by causing anorexia and thus

TNF- $\alpha$  was called cachectin.

There is, therefore, a necessity in finding out ways to eliminate or antagonize endogenously formed or exogenously administered TNF or, alternatively, to find ways to induce specifically only some of the many effects of TNF or restrict its effects to specific kinds of target cells. Our first attempt in this direction was the development of monoclonal antibodies which neutralize the TNF- $\alpha$  cytotoxic activity and were shown to protect mice against the lethal effect of TNF- $\alpha$  under conditions mimicking elicitation of septic shock, as described in Patent Application No. 73883 of the applicants.

Along the sequence of molecular events which take part in cell response to TNF, the most readily accessible to modulation is the initiation of this process, triggered by the binding of TNF to its cell surface receptors. One way to modulate this interaction is through induced changes in the expression of the TNF receptors. A different way of modulating the binding of TNF to its receptors is through the protective effect of the proteins TBP-I and TBP-II which bind TNF and thus decrease its availability to the TNF receptors.

The present invention provides antibodies specific for the two TNF-binding proteins TBP-I and TBP-II which have the following properties:

- a) they block the effect of TNF on specific cells. This blocking effect is obtained using the antibodies or F(ab) fragments thereof, taking advantage of the ability of said antibodies to

block the binding of TNF to cells, apparently through interaction with structurally related cell surface TNF-binding proteins, i.e. TNF receptors; and

- b) they mimic certain effects of TNF on specific cells. This mimicking effect of the antibodies is most likely due to activation of the receptors for TNF, upon their juxtaposition by the divalent antibodies, in a way which is similar to their activation by TNF itself.

The invention also comprises salts, functional derivatives and active fractions of the antibodies and of the F(ab) fragments thereof.

As used herein, the term "salts" refers both to salts of carboxyl groups and to acid addition salts of amino groups of the protein molecule.

"Functional derivatives" as used herein covers derivatives which may be prepared from the functional groups which occur as side chains on the residues or the N- or C- terminal groups, by means known in the art.

As "active fractions" of the antibodies and their F(ab) fragments, the present invention covers any fragment or precursors of the polypeptide chain of said protein molecules alone or together with associated molecules or residues linked thereto, e.g. sugar or phosphate residues, or aggregates of the protein molecule or the sugar residues by themselves.

The antibodies of the present invention provide a new approach for the modulation of the TNF activity. They may be used both to inhibit and to mimic effects of TNF on specific subsets of cells, depending on the molecular form of the antibodies, specifically on their valence: monovalent forms of the antibodies (e.g. F(ab) fragments) being inhibitory and multivalent forms being able to mimic at least part of the effects of TNF.

The functional interaction of the antibodies of the present invention with cells provides also a new diagnostic tool, based on immunoassays such as radioimmunoassay, ELISA etc., for the detection of over- or under-production of TBPs by cells in the body in certain disorders. In an inverse approach, antibodies against the TBPs, when produced endogenously in the body, will be measured with the use of purified TBPs. Detecting such autoantibodies, when formed in certain autoimmune disorders, is of extreme importance, since their ability to mimic or inhibit the effects of TNF surely has far-reaching bearing on the pathological syndromes of said disorders.

The antibodies may be either polyclonal or monoclonal. They may be raised in rabbits, mice or other animals or tissue cultured cells derived thereof or can be products of cells of human origin. They may also be produced by recombinant DNA technology either in a form identical to that of the native antibody or as chimeric molecules, constructed by recombination of antibody molecules of man and animal origins or in other forms chosen to make the antibodies most suitable for use in therapy.



For the preparation of the antibodies, either purified TBP-I or TBP-II or one or more synthetic peptides identical to the known sequences of each of the proteins, e.g. to the N-terminal protein sequences, may be used to immunize the animals. A further possibility is to fuse one of the possible nucleotide sequences deduced from the amino acid sequence of TBP-I or TBP-II to the gene coding for Protein A and to express the fused Protein A - TBP-I or Protein A-TBP-II gene in E. coli. The fused protein obtained is purified by affinity chromatography on IgG Sepharose column and then used to immunize the animals.

The monoclonal antibodies of the present invention are prepared using conventional hybridoma technique (Kohler et al., Nature 256:495, 1975; Kohler et al., Eur. J. Immunol. 6:511, 1976). After immunization, spleen cells alone or together with lymph node cells of the immunized animals are isolated and fused with a suitable myeloma cell line. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium and then cloned. The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding TBP-I or TBP-II. After identification, the desired clones are grown in bulk, either in suspension culture or in ascitic fluid, by injecting the cells into the peritoneum of suitable host mice. The monoclonal antibodies produced by the hybridomas are then isolated and purified.

The antibodies of the invention are found to inhibit the binding of TNF to cells. Antibodies to the two TBPs affect differently cells of different lines. Antisera to TBP-I block the binding of TNF to the

cervical carcinoma HeLa cells and to the breast carcinoma MCF7 cells, but not to the histiocytic lymphoma U937 cells. Antisera against TBP-II have the inverse specificity. In the chronic myeloid leukemia K562 cells both antisera have inhibitory effects. These observations suggest that the TBPs are structurally related to the cell surface receptors for TNF and therefore that antisera against the TBPs can interact with the cell surface receptors in a way which can block the binding of TNF to cells. Furthermore, they indicate that there are two different TNF receptors and that cells of different lines express these two receptors in different proportions.

Consistently with these notions, F(ab) fragments of the anti-TBP-I antibodies are found to block the effect of TNF on cells of a line to which TNF binding is blocked by the antiserum comprising said antibodies. However, the intact antibody molecules are found to exert the inverse effect; they by themselves elicit in cells a cytotoxic effect identical to the effect induced by TNF. This ability of antibodies apparently directed against cell surface receptors to mimic the effect of the agonist, as opposed to the lack of ability of the F(ab) fragments of the antibody to do so, is mostly a consequence of the ability of the divalent antibody molecule to cause clustering of the receptor molecules. This clustering somehow results in activation of the receptors in a way which is similar or identical to the activation of the receptors by TNF itself. Whatever the mechanisms involved, the fact that the antibodies can either mimic or inhibit the function of TNF, depending on the exact molecular form of the antibody applied, implies that these antibodies can practically serve as inhibitory or mimicking agents to TNF. Furthermore, since the

antibodies to TBP-I and TBP-II bind to two different receptors for TNF, which are expressed on different cells exhibiting different responses to TNF and perhaps having also a different function even when expressed in the same cell, these inhibitory and mimicking effects of the antibodies to TBP-I and TBP-II can be applied for modulating the response to TNF, i.e. augmenting specifically beneficial effects and suppressing specifically deleterious effects of this cytokine.

The invention will now be illustrated by the following examples.

#### Example 1: Purification of the TNF binding proteins

Affinity purification on a column of immobilized TNF. Proteins of pooled urine of healthy postmenopausal women were concentrated 200 fold as described in Patent Applications No. 83878 and No. 90339. A sample of 250 ml of the concentrate was applied to a column constructed of 0.5 ml Affigel-10 to which 3.5 mg recombinant human TNF- $\alpha$  were coupled. The column was then washed with PBS (phosphate buffered saline) till all unbound proteins were removed and the bound proteins were eluted by applying a solution containing 25 mM citric acid, 100 NaCl, 0.02% sodium azide at pH 2.5.

Reverse phase HPLC. The proteins eluted from the TNF affinity column were applied to an Aquapore RP300 column preequilibrated with 0.3% aqueous trifluoroacetic acid. The column was washed with the above solution at a flow rate of 0.5 ml/min, until all unbound protein was removed and then eluted with a gradient of acetonitrile concentrations in 0.3% aqueous trifluoroacetic acid. Two active components were

obtained: TBP-I eluted between 26% and 29% acetonitrile and TBP-II between acetonitrile concentrations of 29% and 32%.

Example 2: Preparation of the polyclonal antibodies against TBP-I and TBP-II

Immunization of rabbits with TBP-I and TBP-II obtained in Example 1 was, at doses of 5 µg and 20 µg respectively, in the following schedule: The proteins were first injected subcutaneously as emulsion in complete Freund adjuvant. Three weeks later they were injected again, intramuscularly, as emulsion in incomplete Freund adjuvant and then twice again subcutaneously as solution in PBS, at one week intervals. The rabbits were bled 10 days after the last immunization.

For the purification of immunoglobulins from the rabbit serum, saturated ammonium sulfate was added to 10ml serum to a final concentration of 50% saturation. After overnight incubation at 4°C, the immunoglobulins were precipitated by centrifugation. The pellet was washed twice with 50% ammonium sulfate, then solubilized in 10mM sodium borate 0.02% sodium azide at pH 9. The solution was then dialyzed extensively against the borate-azide solution. It was then applied for chromatography on HPLC Mono-Q column, from which the proteins were eluted with a gradient of 0-500 mM NaCl in the above borate-azide solution. The immunoglobulins eluted at a salt concentration of approximately 70 mM NaCl.

The antiserum to TBP-I inhibited specific binding of radiolabelled TNF to HeLa cells by about 50% at a dilution of 1:6400. The antiserum to TBP-II suppressed the binding of <sup>125</sup>I-TNF to U-937 cells by about 50%

at similar dilution.

Example 3: Assays for the polyclonal rabbit antibodies against TBP-I and TBP-II

Western blotting analysis. Tested proteins were applied to SDS PAGE on 10% acrylamide gels and then blotted electrophoretically to a nitrocellulose sheet. The nitrocellulose sheet was incubated with 10% milk (v/v) in PBS, then briefly rinsed in PBS and further incubated with the tested antibodies in a multi-lane device. After incubation with either  $^{125}\text{I}$ -labelled Protein A or  $^{125}\text{I}$ -goat antimouse F(ab) fragments of IgG, both at  $5 \cdot 10^5$  CPM/ml, the unbound material was washed and the nitrocellulose sheet was exposed to autoradiography.

Figure 1 shows the Western blot analysis of the binding of antisera against TBP-I and TBP-II to the two proteins. TBP-I (A: lanes 1-6) and TBP-II (B: lanes 1-6) were applied to SDS PAGE at  $2 \mu\text{g}/\text{lane}$  together with  $2 \mu\text{g}$  BSA. Following electrophoresis the proteins were blotted electrophoretically to a nitrocellulose sheet which was then incubated with antiserum to TBP-I (lanes 1-3) or to TBP-II (lanes 4-6) at the following dilutions: lanes 1,4 - 1:100; lanes 2,5 - 1:500; lanes 3,6 - 1:2500. After incubation with the antibodies, the nitrocellulose sheet was incubated with  $^{125}\text{I}$ -labelled protein A and then washed and exposed to autoradiography.

ELISA (Enzyme-linked immunosorbent assay). Ninety-six well ELISA plates were coated either with TBP-I or TBP-II or, as a control, with BSA (bovine serum albumin) at  $1 \mu\text{g}/\text{ml}$  in PBS containing sodium azide by incubation. The wells were then rinsed with PBS-Tween, incubated

with a solution of 0.5% BSA in PBS-Tween, and rinsed again and further incubated with the tested antibodies. After a further rinse, they were incubated with purified goat antibody to rabbit IgG, conjugated to horseradish peroxidase. The activity of horseradish peroxidase-conjugated antibody which bound to the plate was determined colorimetrically at 600 nm.

Figure 2 shows the results of ELISA for the binding of antisera against TBP-I and TBP-II to the two species of TBP. The binding of ( $\square$ ) antiserum against TBP-I to TBP-I, ( $\blacksquare$ ) antiserum against TBP-I to TBP-II, (o) antiserum against TBP-II to TBP-I, and ( $\bullet$ ) antiserum against TBP-II to TBP-II, is presented in terms of the absorbance of the color product of the enzymatic reaction applied in this test. The readings in a control test at which the antibodies were applied on wells coated with BSA were subtracted.

Both Figures 1 and 2 show that TBP-I and TBP-II are immunologically distinct (the slight binding of the antiserum against TBP-II to TBP-I, observed in Fig. 2, could be shown to be due to contamination of the antiserum with antibodies to TBP-I, at low amounts, due to the presence of some TBP-I in the preparation of TBP-II used for immunization).

#### Example 4: Effects of the polyclonal antibodies on binding of TNF to cells

The antisera to TBP-I and TBP-II were diluted in Dulbecco's balanced salt solution (PBS+) containing 0.5% BSA and 0.1% sodium azide (PBS/BSA) and then either directly or, in competition experiments,

after incubation with a sample of TBP, applied for 2 h on the tested cells of the HeLa, MCF7, K562 and U937 cell lines. The cells were then rinsed and tested for binding of TNF.

Figure 3 shows the inhibition of the binding of radiolabelled TNF to U937, K562, HeLa and MCF7 cells with antisera to TBP-I (o) and TBP-II (□). The net binding observed in the absence of antisera (100%) was in U937 cells - 2500 CPM, in K562 cells - 1500 CPM, in HeLa cells - 2400 CPM and in MCF7 cells - 1100 CPM. The results demonstrate that antisera against TBP-I and TBP-II interfere with the binding of TNF to cells; each affecting to different extent cells of different lines. The antiserum against TBP-I inhibits effectively the binding of TNF to HeLa and MCF7 cells, but has no effect on the binding of TNF to U937 cells and only little effect on the binding of TNF to K562 cells. Inversely, the antiserum against TBP-II blocks effectively the binding of TNF to the K562 and U937 cells, but inhibits the binding of TNF to the HeLa and MCF7 cells only at high concentrations. The effect of the antiserum against TBP-II on the latter cells could be shown, by competition experiments, at which pure TBP-I and TBP-II were added to the serum, to be due to the presence of contaminating antibodies to TBP-I in this preparation of antiserum to TBP-II.

#### Example 5: F(ab) fragments of polyclonal antibodies to TBP-I

Immunoglobulins purified from the antiserum to TBP-I were exposed to digestion with papain, in the presence of cysteine/EDTA at an enzyme/substrate ratio of 1:100. The antibody digest was dialyzed against 10mM sodium acetate buffer, pH 5.5 and subjected to cation exchange HPLC on a Mono-S column. The bound proteins were eluted from

the Mono-S column with a 0-300mM gradient of NaCl in 10mM sodium acetate buffer, which resulted in purification of the F(ab) fragments to homogeneity. Purity was verified by SDS PAGE analysis under reducing and non-reducing conditions. To test the effect of these fragments on the cytotoxic activity of TNF, they were applied on HeLa cells, the cells were then rinsed to remove all unbound antibodies and exposed for 10 h to TNF, together with cycloheximide (25 µg/ml). Viability of cells was then determined by the neutral red uptake method.

In Fig. 4: Protection from TNF cytotoxicity by the monovalent fragments of the antibodies to TBP-I: HeLa cells which were pretreated with the F(ab) fragments (3µg/ml) (●) and, for comparison, cells treated in the same way with medium alone, (○) were further incubated for 10 h with TNF, at various concentrations, together with CHI (25 µg/ml). The results show that the F(ab) fragments of the antibodies to TBP-I protect HeLa cells from the cytotoxic effect of TNF - a reflection of the fact that they interfere with the binding of TNF to the TNF receptors expressed on the surface of these cells.

Similar results were obtained when the F(ab) fragments were applied on SV80 cells as illustrated in Fig. 9C, described in detail under Example 6, section f, below.

Table I shows that in the intact state antibodies to TBP-I have the inverse effect to that mediated by their F(ab) fragments; namely, they mimic the effect of TNF and are by themselves cytotoxic.



Table I

TNF-like cytotoxic effect of antibodies to TBP-I

<u>TNF (u/ml)</u>	<u>Cell Viability (%)</u>
--	100
1	31
10	15
100	6
1000	3
anti TBP serum	
(dilution)	
1:6400	69
1:1600	37
1: 400	17
1: 100	9

TNF and the antiserum to TBP-I were applied on SV80 cells for 12 h together with cycloheximide. Viability of the cells was then determined by the neutral red uptake method (see also Fig. 5).

Example 6: Determination of bioactivities of the antibodies to TBP-I

a. Cytotoxic activity

Cells were seeded 24 hr prior to assay in 96 well (microtiter plates) ( $3 \times 10^4$  cells/well). The antibodies to TBP-I or TNF were applied in serial dilutions either in the presence or the absence of cycloheximide (CHI) (25  $\mu$ g/ml in the case of the HeLa cells and 50  $\mu$ g/ml for the other cells). After an incubation period of 12 hr for the HeLa cells and 16 hr for all the others, cell viability was determined by the neutral-red uptake method. Cell viability is presented as the per cent ratio of the viability of cultures incubated with CHI alone (for cells tested in its presence) or without additives.

Figure 5 shows the cytotoxic effect of the antibodies to TBP-I (A) and of TNF (B) on SV80 (○,●) HeLa (□,■), FS11 (△,▲) and HEP-2 (◇,◆) cells.

The antibodies and TNF were applied for 16 hr (12 hr for HeLa cells) together with CHI (25  $\mu\text{g/ml}$  for HeLa and 50  $\mu\text{g/ml}$  for all other cells). Cell viability was quantitated by measuring the uptake of neutral red dye. Viability of cells incubated with anti TBP-I at 1:200 in the absence of CHI was 99% in the SV80 cells, 97% in HeLa cells, 98% in FS11 cells and 96% in the HEP-2 cells. Normal rabbit serum in the range of concentrations of anti-TBP-I applied in this study had no effect in this experiment or in any of the other experiments presented below. All tests were performed in duplicates.

#### b. Induction of PGE<sub>2</sub> synthesis

Cells were seeded in 96-well microtiter plates ( $5 \times 10^4$  cells/well). Ten hours thereafter TNF and the antibodies were applied in serial dilutions. After further incubation for 15 hr at 37°C, the cell growth medium was collected and replaced with fresh medium containing arachidonic acid ( $5 \times 10^{-5}\text{M}$ ). One hour later, the medium was collected again. The PGE<sub>2</sub> content of all samples was determined by an immunoassay as described by Kaever et al., Prostaglandins, 35: 885-902, 1988. Figure 6 shows the enhancement of PGE<sub>2</sub> synthesis by the antibodies to TBP-I and by TNF in HeLa, HEP-2 and FS11 cells and its augmentation by arachidonic acid (AA, 5  $\mu\text{M}$ ).

#### c. Stimulation of fibroblast growth

Growth stimulation in fibroblasts due to TNF and the antibodies to TBP-I was determined essentially as described by Vilcek et al., J. Exp. Med., 163:632-643, 1986. Human foreskin fibroblasts (strain FS11, passage 10-12) were seeded in 96-well microtiter plates ( $10^4$  cells/well). Either TNF, or the antibodies to TBP-I, were applied 18

h later. The rate of thymidine incorporation into the cells after 3 days of further incubation was determined by applying  $^3\text{H}$ -thymidine to the cells (1  $\mu\text{Ci}/\text{well}$ ) and incubating them for another 16 h. The cells were then rinsed once with cold PBS, detached with trypsin, and the amount of label incorporated was determined by harvesting the cells onto glass fiber filters followed by liquid scintillation counting.

Figure 7 shows the growth stimulatory effect of the antibodies to TBP-I (A) and of TNF (B) on human fibroblasts and its reversion by IFN-gamma. Human foreskin fibroblasts (strain FS11) were incubated for 3 days with the antibodies to TBP-I ( $\square$ ) or with TNF ( $\circ$ ), in the presence ( $\bullet, \bullet$ ) or absence ( $\square, \circ$ ) of IFN-gamma (250 U/ml). At the end of this incubation period the rate of  $^3\text{H}$ -thymidine incorporation was determined.

#### d. Inhibition of chlamydial growth

The effect of the antibodies to TBP-I on growth of *Chlamydia trachomatis* (L<sub>2</sub>434/Bu) in the HEP-2 cells was determined, as described for the antichlamydial effect of TNF by Shemer-Avni et al., Infect. Immun. 56:2503-2506, 1988. The antibodies were applied on the HEP-2 cells at the indicated concentration either alone or together with IFN-gamma; first, 1 day before infection with the chlamydiae and again, at the same concentrations, immediately after infection. The yield of chlamydiae 2 days after infection was determined using an immunoperoxidase assay for the chlamydial antigens and expressed as inclusion forming units/ml (IFU/ml).

Figure 8 shows the antichlamydial effect of the antiserum to TBP-I, enhancement of the effect by IFN-gamma and its abolition at high tryptophan concentration. The effect of the antiserum, at the indicated dilutions, in the presence (●) or absence (○) of IFN-gamma (2 U/ml) was quantitated. Increase of tryptophan concentration, at the time of infection of the cells treated with anti-TBP-I from 10 µg/ml to 200 µg/ml abolished the antichlamydial effect (▲).

e. Antibodies to TBP-I have TNF-like effects

Applying the polyclonal antibodies to TBP-I on cells in the presence of the protein synthesis inhibitor cycloheximide (CHI) resulted, within a few hours, in extensive cytolysis. The cytotoxic effect was complement-independent (data not shown) and appeared morphologically very similar to the cytotoxic effect of TNF. It resembled the effect of TNF in several other respects as well:

- (i) The sensitivity of different cell lines to polyclonal anti-TBP-I antibodies followed a similar pattern as their sensitivity to TNF. Thus, human foreskin fibroblasts (strain FS11) and HEp-2 cells, which are relatively resistant to TNF toxicity, were also much less sensitive to the toxicity of the antibodies (Fig. 5).
- (ii) Like TNF, the antibodies failed to kill HeLa and SV80 cells in the absence of protein synthesis inhibitors (see cytotoxic activity under Section a above).
- (iii) The sensitizing effect of protein synthesis inhibitors to the

antibody-mediated cell killing was largely dependent on the timing of their application. Maximal cytotoxicity could be observed when the inhibitors and the antibodies were applied simultaneously. Application of the inhibitors a few hours after the antibodies resulted in significantly reduced cell death (Table II). The same time-dependence was observed for the sensitization by such inhibitors to the cytotoxic effect of TNF (Table II). Thus the antibodies, like TNF, could be either cytotoxic to cells or induce in them resistance to their own toxicity, depending on whether they were applied in the presence or absence of protein synthesis inhibitors.

- (iv) Resistance to both the toxicity of the antibodies and of TNF was induced in the SV80 cells also by pretreatment with IL-1 (Table III). Furthermore, the antibodies and TNF could induce in these cells cross resistance to each other's toxicity (Table III).

Table II

Time-dependent sensitization of SV80 cells to the cytotoxic effect of TNF or of antibodies to TBP-I, by CHI.

Time of CHI application	TNF(100u/ml)	$\bar{a}$ TBP-I (1:200)
Cell viability (%)		
Simultaneously with $\bar{a}$ TBP-I/TNF	<1	<1
+ 1 h	<1	<1
+ 3 h	41	48
+ 6 h	77	73
Not added	100	100

SV80 cells were incubated for 16 h with TNF or with the antibodies to TBP-I ( $\bar{a}$ ). CHI (50  $\mu$ g/ml) was added to the culture at time zero or at 1, 3, or 6 h after application of TNF or of the antibodies ( $\bar{a}$ ). Cell viability was determined at the end of the incubation period by the neutral red uptake method.

Table III

Induction of resistance to the cytotoxic effects of  $\bar{a}$  TBP-I, or of TNF by TNF itself,  $\bar{a}$  TBP-I and IL-1

Treatment	—	Pretreatment (for 6 h)		$\bar{a}$ TBP-I (1:200)
		TNF (100 U/ml)	IL-1 (10 U/ml)	
Cell viability (%)				
CHI (50 $\mu$ g/ml)	100	91	95	85
TNF (10 <sup>4</sup> U/ml) + CHI	8	82	82	71
$\bar{a}$ TBP-I (1:200) + CHI	9	89	84	N.D.

N.D. Not determined

SV80 cells were treated for 1 h with TNF, IL-1,  $\bar{a}$  TBP-I or without additives and incubated further for 6 h in medium alone to allow full recovery of the TNF receptors (pretreatment). The cells were then incubated for 12 h with TNF or with  $\bar{a}$  TBP-I in the presence of CHI, or with CHI alone (treatment). While without any pretreatment, most cells were killed when incubated with TNF or with  $\bar{a}$  TBP-I together with CHI, cells which were first incubated with TNF, IL-1 or  $\bar{a}$  TBP-I in the absence of CHI were largely resistant to subsequent treatment with TNF or  $\bar{a}$  TBP-I in the presence of CHI.

Further examination of the effect of antibodies to TBP-I, when applied on cells in the absence of protein synthesis blockers, revealed that under these conditions the antibodies mediated several non-cytocidal TNF-like effects. In the foreskin fibroblasts and HEp-2 cells, which are quite resistant to TNF-cytotoxicity, as well as in the TNF sensitive HeLa cells, the antibodies, similarly to TNF had a marked stimulatory effect on the synthesis of PGE<sub>2</sub> (Fig. 6). The effect of both was particularly prominent when arachidonic acid was added to the cells, suggesting that in both cases it reflects an increase, not in the release of arachidonic acid, but in its conversion to prostaglandin. An additional TNF-like effect of the antibodies in the foreskin fibroblasts was an enhancement of the incorporation of thymidine (Fig. 7), apparently reflecting stimulation of cell growth. Like the growth stimulatory effect of TNF, the stimulation of fibroblast growth by the antibodies was obliterated when the cells were treated simultaneously with IFN-gamma (Fig. 7).

In HEp-2 cells, TNF suppresses the growth of chlamydiae, obligate parasitic bacteria which grow intracellularly within membrane bound structures. As shown in Fig. 8, growth of chlamydiae in these cells was also markedly inhibited by the antibodies to TBP-I. Inhibition of chlamydial growth by TNF is synergistic with the antichlamydial effect of IFN-gamma and is largely abrogated when the HEp-2 cells are grown in the presence of increased concentrations of tryptophan. The antichlamydial effect of the antibodies to TBP-I was affected by IFN-gamma and tryptophan in a similar manner (Fig. 8).

f. The TNF-like activity of the antibodies to TBP-I correlates with their ability to crosslink the TNF receptor molecules

To explore the mechanisms for the TNF-like activity of antibodies to TBP-I, we tested the effect of monovalent F(ab) fragments of anti-TBP-I on cell function. Like the intact antibodies, the monovalent fragments effectively blocked the binding of radiolabelled TNF to cells, suggesting that they maintained the ability to bind to the cell surface TNF receptors ( $EC_{50}$  was about 0.8  $\mu\text{g/ml}$  for the intact antibodies and 1  $\mu\text{g/ml}$  for the monovalent fragments). However, while in their intact form the antibodies were cytotoxic to CHI-treated SV80 cells at concentrations as low as 0.1  $\mu\text{g/ml}$ , the monovalent F(ab) fragments of the antibodies did not exhibit any toxic effects (Fig. 9A). Indeed, by virtue of their ability to inhibit the binding of TNF to cells, the monovalent F(ab) fragments not only failed to kill the SV80 cells, but even had some inhibitory effect on their killing by TNF (Fig. 9C).

To check whether this loss of TNF-like activity in the fragmented antibodies was related to their monovalence we performed experiments to determine whether cross-linking of the F(ab) fragments would result in resurgence of their cytotoxic activity. It has been shown before that pulse-treatment of SV80 cells with TNF at 4°C, followed by incubation with CHI at 37°C, is sufficient to cause effective cell death. The intact antibodies to TBP-I were also cytotoxic under these conditions (compare solid and empty circles in Fig. 9A), while monovalent F(ab) fragments were not cytotoxic. However, when the F(ab)-pretreated cells were treated subsequently with goat antibodies to rabbit Ig to elicit cross-linking of the cell-bound antibody



fragments, extensive cell death occurred (Figs. 9B, 10).

Figure 9A shows the lack of cytotoxic activity of monovalent F(ab) fragments of the antibodies to TBP-I ( $\diamond$ ), and the cytotoxic effect of the anti-TBP-I immunoglobulins (o) at different concentrations, when applied to SV80 cells for 16 h together with CHI (50  $\mu$ g/ml), and of "pulse" treatment with the anti-TBP-I immunoglobulins ( $\bullet$ ). Titration of the cytotoxic effect of "pulse" treatment with the anti-TBP-I immunoglobulins was performed as follows: The cells were incubated with the antibodies, at the indicated concentrations, for 2 h at 4°C, rinsed and incubated further at 37°C with CHI (50  $\mu$ g/ml) with no further addition of the antibodies.

Figure 9B shows the effect of goat antibodies to rabbit immunoglobulins when applied to SV80 cells which had been "pulse" treated with monovalent F(ab) fragments of the anti-TBP-I ( $\bullet$ ) or to untreated cells (o) (5  $\mu$ g/ml). The "pulse" treatment with the F(ab) fragments was performed as described above for the intact immunoglobulins in Figure 9A.

Figure 9C shows the protection from TNF cytotoxicity by the monovalent fragments of the antibodies to TBP-I: SV80 cells which were "pulse" treated with the F(ab) fragments, as in Figure 9B and, for comparison, cells treated in the same way with medium alone were further incubated for 16 h with TNF, at various concentrations, together with CHI (50  $\mu$ g/ml).

Figure 10 shows the morphology of SV80 cells after "pulse" treatment

with monovalent F(ab) fragments of anti-TBP-I and further incubation in the presence or absence of anti immunoglobulin antibodies. Anti TBP-I immunoglobulins and their monovalent F(ab) fragments were applied to the SV80 cells for 2 hr in the cold at a concentration of 3 µg/ml followed by rinsing and incubation for 16 hr at 37°C in the presence of CHI with or without goat anti-rabbit IgE (4.5 µg/ml). All other conditions of the assay were as for Fig. 7. Photographs were taken at a magnification of x125 after staining the cells with neutral red.

#### Example 7: Monoclonal antibodies to TBP-I

##### Production of the monoclonal antibodies

Female Balb/C mice (8 weeks old) were injected with 1 µg purified TBP-I in an emulsion of complete Freund's adjuvant into the hind foot pads, and three weeks later, subcutaneously into the back in incomplete Freund's adjuvant. The other injections were given in weekly intervals, subcutaneously in PBS. Final boosts were given 4 days (i.p.) and 3 days (i.v.) before the fusion with 9.0 µg of TBP-I in PBS. Fusion was performed using NSO/Mr cells and lymphocytes prepared from both the spleen and the local lymphocytes of the hind legs as fusion partners. The hybridomas were selected in DMEM supplemented with HAT, 15% horse serum and gentamycin 2 µg/ml. Hybridomas that were found to produce antibodies to TBP-I were subcloned by the limiting dilution method and injected into Balb/C mice that had been primed with pristane for the production of ascites. Immunoglobulins were isolated from the ascites by ammonium sulfate precipitation (50% saturation) and then dialyzed against PBS containing 0.02% azide. Purity was approximately 60% as estimated by

analysis on SDS-PAGE and staining with commassie blue. The isotypes of the antibodies were defined with the use of a commercially available ELISA kit (Amersham, U.K.).

Several positive monoclonal antibodies were obtained and 7 of them selected for further studies. The clones producing them were designated clones 17, 18, 20, 23, 30, 34 and 68. The isotypes and other characteristics of the monoclonal antibodies are given in Fig. 11.

The clones were subcloned for further studies. Some of the isolated subclones with their isotype and binding of TBP-I in inverted RIA are listed in Table IV.

Hybridomas TBP-I 18-1 and TBP-I 34-6 were deposited with the Collection Nationale de Cultures de Microorganismes, Institut Pasteur (CNCM), 25, rue du Docteur Roux, 75724 Paris CEDEX 15, France on March 12, 1990 and were assigned No. I-926 and No. I-927, respectively.

#### Example 8: Monoclonal antibodies to TBP-II

Monoclonal antibodies to TBP-II were produced in the same way as the monoclonal antibodies to TBP-I, after immunization of mice with TBP-II. Several positive clones were isolated, subcloned, and characterized. Some of the isolated subclones are listed in Table V.

Hybridomas TBP-II 13-12 and TBP-II 70-2 were deposited with the CNCM on March 12, 1990 and were assigned No. I-929 and No. I-928, respectively.

Table IV

Subclones producing monoclonal antibodies to TBP-I

Clone number	Screening with iRIA [CPM]	Screening of subclone with iRIA [CPM]	Isotype
7.5	10000	2900	IgG <sub>2a</sub>
.8		2800	IgG <sub>2a</sub>
.9		5200	IgG <sub>2a</sub>
8.1	13440	10800	IgG <sub>1</sub>
.3		11300	IgG <sub>1</sub>
.11		11000	IgG <sub>1</sub>
13.2	28300	13800	IgG <sub>2a</sub>
.3		15000	IgG <sub>2a</sub>
.5		12900	IgG <sub>2a</sub>
14.6	17000	5400	IgG <sub>2b</sub>
.7		6200	IgG <sub>2b</sub>
.13		5700	IgG <sub>2b</sub>
16.1	29000	18000	IgG <sub>2a</sub>
.3		16000	IgG <sub>2a</sub>
.7		17500	IgG <sub>2a</sub>
17.2	7076	2100	IgG <sub>2a</sub>
.7		2100	IgG <sub>2a</sub>
.9		2200	IgG <sub>2a</sub>
18.1	28000	28800	IgG <sub>2b</sub>
.2		27200	IgG <sub>2b</sub>
.3		29800	IgG <sub>2b</sub>
20.2	46000	29800	IgG <sub>2a</sub>
.5		32800	IgG <sub>2a</sub>
.11		31400	IgG <sub>2a</sub>
23.1	5300	1700	IgG <sub>2a</sub>
.3		1500	IgG <sub>2a</sub>
.5		1900	IgG <sub>2a</sub>
29.1	4900	1600	IgG <sub>2a</sub>
.4		1200	IgG <sub>2a</sub>
.5		1100	IgG <sub>2a</sub>
30.1	48000	26700	IgG <sub>2a</sub>
.3		27500	IgG <sub>2a</sub>
.5		26700	IgG <sub>2a</sub>

Table IV cont.

Subclones producing monoclonal antibodies to TBP-I

Clone number	Screening with iRIA [CPM]	Screening of subclone with iRIA [CPM]	Isotype
34.6	29000	32800	IgG <sub>2a</sub>
.7		34200	IgG <sub>2a</sub>
.12		32300	IgG <sub>2a</sub>
39.1	4400	31800	IgG <sub>2a</sub>
.6		31700	IgG <sub>2a</sub>
.8		31500	IgG <sub>2a</sub>
68.1	46700	28900	IgG <sub>2a</sub>
.4		28000	IgG <sub>2a</sub>
.6		27500	IgG <sub>2a</sub>
79.6	2100	5000	IgG <sub>2a</sub>
.7		1900	IgG <sub>2a</sub>
.8		1900	IgG <sub>2a</sub>
80.1	2200	1900	IgG <sub>2b</sub>
.4		2100	IgG <sub>2b</sub>
.13		1800	IgG <sub>2b</sub>
83.2	1400	1600	IgG <sub>2a</sub>
.7		1700	IgG <sub>2a</sub>
.9		1600	IgG <sub>2a</sub>
86.1	2300	1500	IgG <sub>2a</sub>
.7		1400	IgG <sub>2a</sub>
.8		1600	IgG <sub>2a</sub>
91.2	1700	1900	IgG <sub>2a</sub>
.3		1500	IgG <sub>2a</sub>
.6		1600	IgG <sub>2a</sub>
92.1	2600	1900	IgG <sub>2a</sub>
.2		1600	IgG <sub>2a</sub>
.6		1500	IgG <sub>2a</sub>

Table V

Subclones producing monoclonal antibodies to TBP-II

Clone number	Screening with iRIA [CPM]	Screening of subclone with iRIA [CPM]	Isotype
13.11	31800	31000	IgG <sub>1</sub>
.12		31500	IgG <sub>1</sub>
.13		31100	IgG <sub>1</sub>
14.1	15300	15400	IgG <sub>2a</sub>
.6		16200	IgG <sub>2a</sub>
.7		15300	IgG <sub>2a</sub>
20.2	12800	14200	IgG <sub>2b</sub>
.5		14300	IgG <sub>2b</sub>
.6		14800	IgG <sub>2b</sub>
22.7	20400	20000	IgG <sub>1</sub>
.8		19300	IgG <sub>1</sub>
27.1	18000	27000	IgG <sub>2a</sub>
.3		25000	IgG <sub>2a</sub>
.9		28000	IgG <sub>2a</sub>
32.4	11315	10900	IgG <sub>2b</sub>
.5		10700	IgG <sub>2b</sub>
.6		11200	IgG <sub>2b</sub>
33.1	18400	11400	IgG <sub>1</sub>
.3		10500	IgG <sub>1</sub>
.4		14800	IgG <sub>1</sub>
36.1	27500	26600	IgG <sub>2a</sub>
.5		24900	IgG <sub>2a</sub>
.6		24900	IgG <sub>2a</sub>
41.3	13800	18100	IgG <sub>1</sub>
.7		18100	IgG <sub>1</sub>
.10		18800	IgG <sub>1</sub>
67.1	16800	10900	IgG <sub>2a</sub>
.16		10800	IgG <sub>2a</sub>
.17		10900	IgG <sub>2a</sub>
70.2	15100	5100	IgG <sub>2a</sub>
.3		5200	IgG <sub>2a</sub>
.4		5300	IgG <sub>2a</sub>
77.2	15300	11800	IgG <sub>2b</sub>

Table V cont.

Subclones producing monoclonal antibodies to TBP-II

Clone number	Screening with iRIA [CPM]	Screening of subclone with iRIA [CPM]	Isotype
78.9	25300	21400	IgG <sub>2a</sub>
82.1	17600	25900	IgG <sub>1</sub>
.4		25700	IgG <sub>1</sub>
.10		26400	IgG <sub>1</sub>
86.2	8800	12200	IgG <sub>2b</sub>
.5		12600	IgG <sub>2b</sub>
.11		12800	IgG <sub>2b</sub>
19.6		29700	IgG <sub>2a</sub>
.9		28900	IgG <sub>2a</sub>

Example 9: Inverted Radioimmunoassay (iRIA) for the detection of the monoclonal antibodies to TBP-I or TBP-II

This assay was used for estimating the level of the anti TBP antibodies in the sera of the immunized mice and for screening for the production of the antibodies by hybridomas. PVC, 96-well microtiter plates (Dynatech 1-220-25) were coated for 12 hr at 4°C with affinity purified goat anti mouse F(ab) immunoglobulins (Biomakor, Israel 10 µg/ml in PBS containing 0.02% NaN<sub>3</sub>), then blocked for 2 hr at 37°C with 0.5% BSA in PBS supplemented with 0.05% Tween 20 (Sigma) and 0.02% NaN<sub>3</sub> (blocking buffer) and washed 3 times with PBS containing 0.05% Tween 20 and 0.02% NaN<sub>3</sub> (washing buffer). Serum samples, in serial dilutions, or samples of hybridoma growth media (50 µl) were applied into the wells for 2 hr at 37°C. The plates were rinsed with washing buffer and <sup>125</sup>I-labelled TBP-I (10,000 cpm, in blocking

buffer) was applied into the wells. After further incubation of 2 hr at 37°C, the plates were washed and the amount of label which bound to individual wells was determined in the gamma-counter.

Example 10: Epitope mapping of TBP-I by cross competition analysis with monoclonal antibodies

Poly vinyl chloride (PVC), 96-well microtiter plates were coated, as described above, with purified monoclonal antibodies (mAbs) to TBP-I (25 µg/ml) and, following rinsing and blocking, samples of <sup>125</sup>I-labeled TBP-I (50,000 cpm per well) which had been pre-incubated for 2 hr, at 37°C, with the same or another monoclonal antibody to TBP-I (at 1 µg/ml) were put into the wells. The plates were incubated overnight at 4°C, washed and the radioactivity bound to each well was determined in the gamma-counter. The results in Fig. 11 are expressed in percent of the control well where TBP-I was allowed to bind in the absence of competing mAbs..

Figure 11 shows the epitope mapping of TBP-I by cross competition analysis with 7 different mAbs to TBP-I and correlation with the ability of the antibodies to suppress binding of TNF to HeLa cells and to mediate TNF-like cytotoxicity. Binding of radiolabelled TBP-I to the mAbs (applied at saturating concentration, 200 ng/ml) in the inverted RIA, cross competition analysis of the mAbs for their binding to TBP-I and determination of the effect of the mAbs on the binding of TNF to HeLa cells were carried out as described. The TNF-like cytotoxicity of the antibodies was measured in two ways. In the first, (-anti Ig), SV80 cells were incubated 16 hr at 37°C with the mAbs in the presence of CHI (50 µg/ml). In the second, (+anti Ig),



the effect of crosslinking by anti-immunoglobulin antibodies on the cytotoxicity of the mAbs was tested. In this case, the SV80 cells were pulse-treated with the antibodies (2 hr at 4°C), rinsed, and further incubated (16 hr at 37°C) with rabbit anti-mouse F(ab)<sub>2</sub> antibodies (+ anti Ig) in the presence of CHI. Cell viability was determined by the neutral red uptake method. The cytotoxic activity of the antibodies is expressed in TNF equivalent units per mg immunoglobulin, where one TNF equivalent unit is defined as the amount of antibody exerting the same cytotoxicity as 1 U/ml TNF (16 pg/ml) (n.ctx - not cytotoxic).

The epitope mapping of TBP-I by cross competition analysis with the 7 mAbs suggests that these antibodies bind to four topologically distinct areas on the molecule (A - defined by mAbs 17 and 23, B - by 18, C - by 20 and 34, and D - by 30 and 68 (Fig. 11). An exceptional competition pattern was observed for the antibodies associated with epitope region D. The antibodies not only competed effectively with each other but also with the antibodies belonging to regions B and C. Moreover, the antibodies defining regions B and C competed, although less effectively, with the antibodies which bind to epitope D. One possible interpretation for this phenomenon is that epitopes B and C, while spatially distinct, overlap both with the epitope region represented by group D. An alternative possibility is that binding of an antibody to the epitope region D imposes a conformational change on TBP-I which prevents the binding of the antibodies recognizing determinants in B and C. The latter hypothesis seems consistent with the fact that the antibodies defining epitope D were the only ones found to recognize TBP-I after its denaturation with SDS in the

presence of  $\beta$ -mercapto ethanol (not shown).

When applied to HeLa cells, the seven monoclonal antibodies to TBP-I described above, as well as ten other monoclonal antibodies to TBP-I had all, a marked inhibitory effect on TNF binding (Fig. 11), implying that all the different epitopes which they recognize in the TBP-I molecule are also present in the cell surface receptors for TNF.

The effect of the antibodies on binding of TNF to HeLa cells was performed as follows: HeLa cells were seeded into 15 mm tissue culture plates at a density of  $2.5 \times 10^5$  cells/well. After 24 h incubation at 37°C in 5% CO<sub>2</sub> atmosphere, the cells were transferred to ice, the growth medium was removed and the antibodies, diluted in PBS containing 0.5% BSA and 0.1% sodium azide, were applied to the cells for 2 h. The cells were then rinsed and tested for binding of TNF.

#### Example 11: TNF-like effect of monoclonal antibodies to TBP-I

To obtain additional information on the molecular requirements needed to trigger TNF-like biological activities with antibodies to TBP-I, we examined whether mAbs against TBP-I mediated TNF-like cytotoxicity. Two experimental approaches were taken: in the first, the antibodies were tested for cytotoxicity on SV80 cells without any further treatment (Fig. 11, -anti Ig) and in the second the cytotoxicity of the mAbs was tested after crosslinking them with anti-mouse immunoglobulin antibodies (Fig. 11, +anti Ig). As noted above, all mAbs tested appeared to bind to the class I TNF receptors, yet only two of the 17 mAbs had mild cytotoxic activity on SV80 cells. This difference in biological activity among the mAbs could not be

correlated with their isotype nor with their binding capacity in the inverted RIA (Fig. 11). It did appear, however, to relate to the site of binding of the antibodies on the receptor molecule. In cross-competition analysis the two cytotoxic antibodies were found to bind to the same epitope region in TBP-I. None of the other antibodies bound to it (A in Fig. 11). Retesting the effect of the antibodies after cross linking them with anti-immunoglobulin antibodies, we found them all to be highly cytotoxic, to an extent which appeared roughly proportional to the effectiveness at which they bound TBP-I (compare in Fig. 11: cytotoxicity + anti Ig to the binding to TBP-I in inverted RIA).

Testing whether different mAbs to TBP-I can supplement each other in mediating TNF-like cytotoxicity we found that mixtures of two mAbs mapping to different epitope regions on TBP-I were highly cytotoxic to SV80 cells. For example, mixtures of the mAb 18 (epitope region B), which is not cytotoxic by itself, with the non-cytotoxic mAb 20 or 34 (both epitope region C) exerted strong cytotoxicity (app.  $3 \times 10^5$  TNF eq. U/mg Ig; see Fig. 11 for definition of unitage). Mixtures of two mAbs mapping to the same epitope region were not more cytotoxic than each mAb alone.

#### Example 12: The Use of anti-TNF binding protein antibodies for affinity chromatography

TNF binding protein antibodies can be utilized for the purification of TNF binding proteins by affinity chromatography, as illustrated below with monoclonal antibodies against TBP-I. The monoclonal antibodies were produced as described in Example 7.

The monoclonal antibodies for affinity chromatography were selected by testing their binding capacity for the radiolabeled antigen in a solid phase radio immunoassay. Ascites from all hybridomas was purified by ammonium sulfate precipitation at 50% saturation followed by extensive dialysis against PBS. PVC 96 well plates were coated with the purified McAbs as described in Example 9. After blocking the plates with PBS containing 0.5% BSA, 0.05% Tween 20 (Sigma) and 0.02%  $\text{NaN}_3$ , the wells were incubated with 50,000 cpm  $^{125}\text{I}$ -TNF for 2 h at 37°C, then washed and the radioactivity which had bound to each well was quantitated in the gamma-counter. The antibodies with the highest binding capacity were examined for their performance in immuno affinity chromatography.

Polyacryl hydrazide agarose was used as resin to immobilize the antibodies. The semipurified immunoglobulins were concentrated and coupled to the resin as specified by Wilchek and Miron, Methods in Enzymology 34:72-76, 1979. Three monoclonal antibodies against TBP-I, clones 16, 20, and 34 were tested in these experiments.

Antibody columns of 1 ml bed were constructed. Before use, all columns were subjected to 10 washes with the elution buffer, each wash followed by neutralization with PBS. Then the columns were loaded with 120 ml of concentrated urinary proteins in PBS with 0.02%  $\text{NaN}_3$ . The flow rate of the columns was adjusted to 0.2 to 0.3 ml per minute. After loading, the columns were washed with 50 ml PBS and then eluted with a solution containing 50 mM citric acid, pH 2.5, 100 mM NaCl and 0.02%  $\text{NaN}_3$ . Fractions of 1 ml were collected. Samples of the applied

urinary proteins, the last portion of the wash (1 ml) and of each elution fraction (8 fractions of 1 ml per column) were taken and tested for protein concentration (Table VI) and activity in the bioassay for TBP I (Table VII). According to the protein measurements before and after coupling of the antibodies to hydrazide agarose, the following amounts of immunoglobulin had been bound to the columns:

Clone 16: 8.4 mg/ml agarose

Clone 20: 7.2 mg/ml agarose

Clone 34: 9.4 mg/ml agarose

All protein measurements were done according to a micro-fluorescamin method in comparison to a standard solution containing 100 µg BSA/ml (Stein, S. and Moschera, J., Methods Enzymol. 79:7-16, 1981).

The results of bioactivity and protein concentrations of applied, last wash and elution fractions are summarized in tables VI and VII.

Table VI

Protein Concentrations

	Clone 16 [ $\mu\text{g/ml}$ ]	Clone 20 [ $\mu\text{g/ml}$ ]	Clone 34 [ $\mu\text{g/ml}$ ]
Applied (120 ml)	35800	35800	35800
Unbound (120 ml)	35700	35700	35700
Last wash (1 ml)	42	38	12
Elution 1 (1 ml)	47	38	45
Elution 2 (1 ml)	550	784	714
Elution 3 (1 ml)	151	150	194
Elution 4 (1 ml)	100	51	122
Elution 5 (1 ml)	32	24	35

The protein concentrations were determined by a micro-fluorescamin method in comparison to a BSA standard (100  $\mu\text{g/ml}$ )

Table VII

Purification of TBP-I with monoclonal antibodies bound to  
hydrazide agarose

	Clone 16 Bioassay [U/ml]	Clone 20 Bioassay [U/ml]	Clone 34 Bioassay [U/ml]
Applied	210	210	210
Unbound	100	60	80
Last wash	* <detect	<detect	<detect
Elution 1	<detect	850	1200
Elution 2	4800	10100	19500
Elution 3	3500	<detect	<detect
Elution 4	2500	<detect	<detect
Elution 5	1000	<detect	<detect

\* Applied, unbound and last wash were assayed at a dilution of 1:20 and higher and the elution fractions at 1:200 and higher. Samples which gave no discernable signal at this dilution appear as "below detection" (<detect) in the table.

### Claims

1. An antibody to a human tumor necrosis factor binding protein designated TBP-I which specifically recognizes said protein.
2. An antibody as claimed in claim 1 which is further characterized in that it blocks the binding of TNF to HeLa and MCF7 cells.
3. An antibody as claimed in claim 1 or 2 further characterized in that it does not block the binding of TNF to U937 cells.
4. An antibody according to claims 1 to 3 which is a monoclonal antibody.
5. An antibody according to claims 1 to 3 which is a polyclonal antibody.
6. F(ab) fragments of an antibody according to any of claims 1 to 5.
7. A monoclonal antibody according to claim 4 produced from a hybridoma formed by fusion of myeloma cells with spleen cells and lymphocytes of mice previously immunized with TBP-I.
8. A monoclonal antibody according to claim 7 produced from hybridoma TBP-I 18-1, deposited in CNCM under designation I-926.



9. A monoclonal antibody according to claim 7 produced from hybridoma TBP-I 34-6, deposited in CNCM under designation I-927.
10. An antibody to a human tumor necrosis factor binding protein designated TBP-II which specifically recognizes said protein.
11. An antibody as claimed in claim 10 which is further characterized in that it blocks the binding of TNF to U937 and K562 cells.
12. An antibody as claimed in claim 10 or 11 further characterized in that it does not block the binding of TNF to HeLa and MCF7 cells.
13. An antibody according to claims 10 to 12 which is a monoclonal antibody.
14. An antibody according to claims 10 to 12 which is a polyclonal antibody.
15. F(ab) fragments of an antibody according to any of claims 10 to 14.
16. A monoclonal antibody according to claim 13 produced from a hybridoma formed by fusion of myeloma cells with spleen cells and lymphocytes of mice previously immunized with TBP-II.
17. A monoclonal antibody according to claim 16 produced from hybridoma TBP-II 13-12 deposited in CNCM under designation I-929.

18. A monoclonal antibody according to claim 16 produced from hybridoma TBP-II 70-2, deposited in CNCM under designation I-928.
19. The use of an antibody according to any of the preceding claims or F(ab) fragments thereof or salts, functional derivatives or active fractions of the antibody or of the fragment thereof, for blocking the binding of TNF to, and inhibiting its effect on cells.
20. The use of an antibody according to any of the preceding claims or F(ab) fragments thereof or salts, functional derivatives or active fractions of the antibody or of the fragment thereof, for the treatment of conditions wherein effects of TNF, either endogenously formed or exogenously administered, are to be antagonized.
21. The use of an antibody according to any of the preceding claims or F(ab) fragments thereof or salts, functional derivatives or active fractions of the antibody or of the fragment thereof, for mimicking beneficial effects of TNF on cells.
22. The use of an antibody according to any of the preceding claims or F(ab) fragments thereof or salts, functional derivatives or active fractions of the antibody or of the fragment thereof for mimicking the cytotoxic effect of TNF.
23. A quantitative assay for antibodies to TBP-I or TBP-II endogenously produced in man in autoimmune diseases, by measuring their binding to the proteins TBP-I or TBP-II, respectively.

24. An immunoassay for the TNF binding proteins TBP-I and TBP-II in body fluids characterized by measuring their interaction with an antibody according to claim 1 or claim 10, respectively.
25. A pharmaceutical composition comprising as active material an antibody or F(ab) fragments thereof or salts, functional derivatives or active fractions of the antibody or of the fragment thereof according to any of claims 1 to 18.
26. A method for the purification of human TNF binding proteins TBP-I or TBP-II utilizing a suitable antibody according to claims 1 to 18 comprising the following steps:
- a. coupling said antibody to a suitable resin to construct an immunoaffinity column;
  - b. loading a solution containing said protein on said immunoaffinity column;
  - c. washing away the non-bound proteins with a suitable washing buffer;
  - d. eluting the bound TNF binding protein TBP-I or TBP-II with a suitable eluent; and
  - e. collecting the enriched fraction of said TBP-I or TBP-II.
27. A method according to claim 26 wherein the resin is hydrazide agarose.
28. A method according to claims 26 and 27 wherein the eluent is a buffered solution of pH=2.5.

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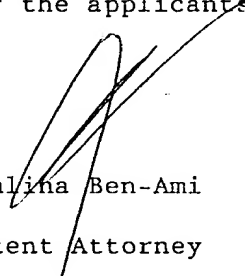
29. A method according to claims 26 to 28 for the purification of TBP-I.

30. A method according to claims 26 to 28 for the purification of TBP-II.

31. Human TNF binding protein TBP-I purified by the method according to claims 26 to 29.

31. Human TNF binding protein TBP-II purified by the method according to claims 26 to 28 and 30.

For the applicants



Paulina Ben-Ami

Patent Attorney

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